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Case Report

An autopsy case of rhabdomyolysis related to vegetamin and genetic analysis of the rhabdomyolysis-associated genes

Aya Matsusue MS (Assistant Professor) [a,*], Kenji Hara PhD (Senior Assistant Professor) [a], Mitsuyoshi Kageura PhD (Professor) [b], Masayuki Kashiwagi MD (Assistant Professor) [a], Wang Lu MD (PhD Student) [c], Akiko Ishigami PhD (Assistant Professor) [d], Takako Gotohda PhD (Assistant Professor) [d], Itsuo Tokunaga PhD (Senior Assistant Professor) [d], Akiyoshi Nisimura MD PhD (Professor) [d], Tomoko Sugimura MD (PhD Student) [a], Shin-ichi Kubo MD PhD (Professor) [a]

[a] Department of Forensic Medicine, Faculty of Medicine, Fukuoka University, Japan
[b] General Medical Research Center, Faculty of Medicine, Fukuoka University, Japan
[c] Department of Human Pathology, The University of Tokushima Graduate School, Japan
[d] Department of Forensic Medicine, The University of Tokushima Graduate School, Japan

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ABSTRACT

We report an autopsy case of a man who died 2 days after taking an overdose of vegetamin. The autopsy findings were as follows: the epidermis on the axillary fossa and the inguinal skin had become macerated. Skeletal muscle was discolored. Concentrations of urea nitrogen, creatinine and urine myoglobin were 1.95 g/day, 0.66 g/day and 1100 ng/mL, respectively. Immunohistochemically, myoglobin was strongly stained at the Bowman's capsule, and tubular lumen and epithelium. 8-OH-dG was strongly stained in renal tubular epithelium in which cell nuclei were strongly stained. ORP-150 was observed in intraglomerular cells and renal tubular epithelium. The concentrations of phenobarbital, promethazine and chlorpromazine ranged from therapeutic to toxic levels, from toxic to lethal levels and toxic level, respectively. His cause of death was considered to be vegetamin-induced rhabdomyolysis. In genetic analysis of this subject, there were two heterozygous silent mutations in the three hot-spot regions in the *RYR1* gene. In the *CPT II* gene, the subject was found to be heterozygous for an amino acid substitution in exon 4, ^{1203}G > A causing a ^{368}Val > Ile amino acid substitution. There was no mutation in the *VLCAD* gene or *CYP2C19* gene. The subject was heterozygous for CYP2D6*1 and CYP2D6*2.

1. Introduction

Rhabdomyolysis is a syndrome caused by injury to skeletal muscles and the resultant leakage of muscle fiber contents into the plasma. Rhabdomyolysis can lead to renal failure, and even death. It was reported that various drugs cause rhabdomyolysis.[1] There is a possibility that rhabdomyolysis can be triggered by fragility of muscular cells or a reduction in the metabolism of the causative agent, which are caused by genetic background. The *Ryanodine receptor1* (*RYR1*) gene, the *carnitine palmitoyltransferase II* (*CPT II*) gene, the *very-long-chain acyl-CoA dehydrogenase* (*VLCAD*) gene and genes encoding vegetamin-metabolizing enzyme were examined as candidates for rhabdomyolysis-susceptibility genes. RYR1, encoded by a gene located on human chromosome 19, is mainly expressed in skeletal muscle where it mediates the release of Ca^{2+} from the sarcoplasmic reticulum, following depolarization of the plasmalemma.[2] It has been reported that the vast majority of variants associated with malignant hyperthermia and central core disease are located in hot-spot regions (exons 2–17, exons 39–46 and exons 85–103) referred to as regions I–III.[3] Two *RYR1* gene mutations (^{401}Arg > Cys and ^{614}Arg > Cys) are associated with malignant hyperthermia, environmental heat stroke and exercise-induced rhabdomyolysis.[4,5] The CPT enzyme system plays an important role in the transfer of long-chain fatty acids from the cytosolic compartment to the mitochondrial matrix, where beta-oxidation occurs.[6] CPT II deficiency is an important cause of recurrent rhabdomyolysis.[7] It has been reported that F383Y[8] was associated with rhabdomyolysis. VLCAD is an enzyme catalyzing the dehydrogenation of long-chain fatty acids in the first step of mitochondrial fatty acid oxidation.[9] Recognized heritable causes of rhabdomyolysis are defects in fatty acid oxidation.[10] Hydroxylation of phenobarbital is attributed to the cytochrome P450 (CYP) enzyme system, primarily CYP2C9, CYP2C19 and CYP2E1.[11]

* Corresponding author. Tel.: +81 92 801 1011x3335; fax: +81 92 801 4266.
E-mail address: miyoshi@fukuoka-u.ac.jp (A. Matsusue).

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Promethazine[12] and chlorpromazine[13] are mostly metabolized by CYP2D6. However, there have been few systemic analyses of those genes in actual cases. We experienced an autopsy case of rhabdomyolysis suspected to have been caused by vegetamin A. Vegetamin A (Shionogi, Osaka, Japan), containing phenobarbital 40 mg, promethazine 12.5 mg and chlorpromazine 25 mg, is an antipsychotic agent, one of the adverse effects of which is rhabdomyolysis. It is not clear that how vegetamin or other drugs cause rhabdomyolysis. We analyzed potential rhabdomyolysis-susceptibility genes in an autopsy case of vegetamin-induced rhabdomyolysis.

2. Case report

A man in his forties and his female cohabiter both took about fifty vegetamin tablets in an attempted suicide. Two days later, she came out of a coma and called for an ambulance, but he had already died.

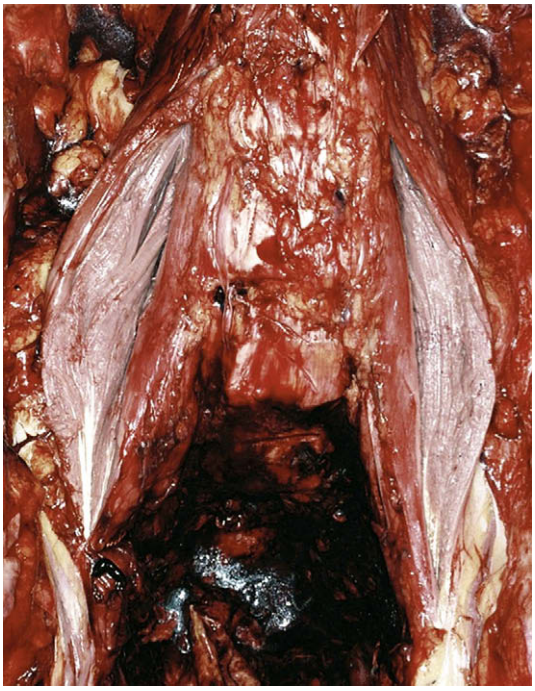

Fig. 1. Autopsy finding: skeletal muscle showed discoloration.

2.1. Autopsy findings

The deceased was 170.5 cm tall and weighed 60.5 kg. The rectal temperature was 15 °C at autopsy (room temperature was 14 °C). The epidermis was removed by maceration in various parts of the body. The back and superolateral epidermis of the left femoral region was easily detached from the underlying dermis on finger touch. Viscous fluid adhered to the lower part of the nose and around the mouth. Internally, the brain was edematous and weighed 1520 g. There was congestion in the subarachnoid space. The heart weighed 390 g and was hypertrophic. Both lungs weighed 1025 g and were edematous and congestive. Skeletal muscle was discolored in the whole body (Fig. 1). The kidney was slightly softened and significantly congested. The left and right kidneys weighed 150.7 g and 134.5 g, respectively. The renal pelvic urine was cloudy yellow and had a strong urine odor. The amount of urine retained in the bladder was 426.4 g, and the color was a slightly red-tinged yellow. Autopsy also revealed his postmortem duration was suspected to be from 1.5 to 3 days.

2.2. Biological examination of urine

Concentrations of urea nitrogen, creatinine and urine myoglobin were 1.95 (standard value; 6.5–13.0) g/day, 0.66 (standard value; 0.70–2.20) g/day and 1100 (standard value; not detected or <10) ng/mL, measured in a clinical laboratory (SRL, Tokyo, Japan), respectively.

Biochemical testing of blood could not be conducted because of severe hemolytic change.

2.3. Histopathological findings

Tissue samples were fixed with phosphate-buffered formalin, embedded in paraffin and sectioned at 4 μm. Hematoxylin–eosin (HE) was used as the conventional stain. Immunostaining of kidney tissue was performed with antibodies against myoglobin (1:800, Dako, Japan), 8-hydroxy-2′-deoxyguanosine (8-OH-dG, 1:200, JICA, Japan), superoxide dismutase Cn/Zn enzyme (SOD, pre-diluted, JICA, Japan) and 50 kDa oxygen-regulated protein (ORP-150, original).[14] The immunostaining was carried out using an ENVISION/AP Kit (Dako, Japan) following the manufacturer's instructions. For 8-OH-dG, SOD and ORP-150 immunostainings, the sections were pretreated by autoclaving (121 °C, 15 min).

HE staining revealed the kidney to be congested, exfoliation of the renal tubule epithelium to the lumen and strongly stained cell nuclei. In liver, mild fatty liver and centrolobular necrosis were

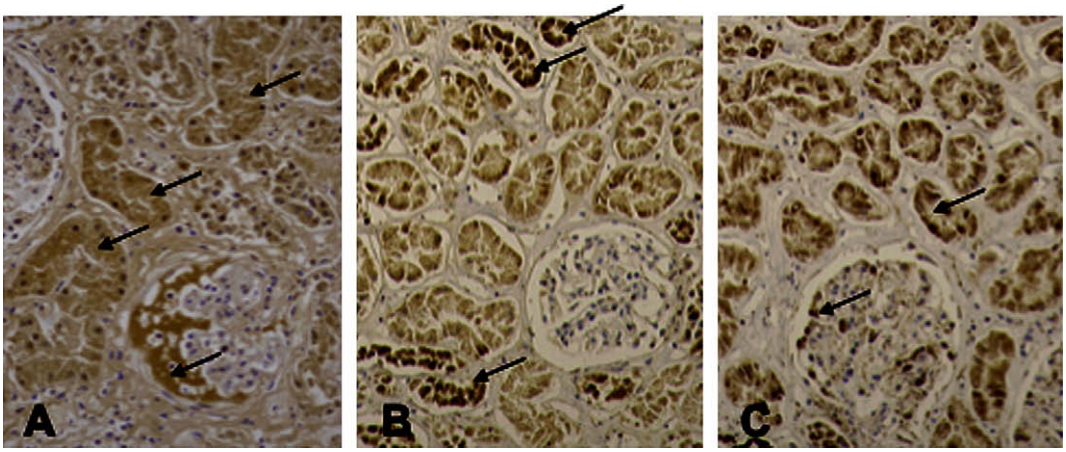

Fig. 2. Immunostaining for myoglobin (A), 8-OH-dG (B) and ORP-150 (C) (20×).

observed. The pancreas partially exhibited colliquative necrosis. Heart was slightly hypertrophic with fibrosis and lipomatosis. Lungs showed severe congestion and edema. Brain was also edematous. Skeletal muscles showed discoloration, but no morphological changes such as edema or degeneration was observed. Immunohistochemically, myoglobin was strongly stained at the Bowman's capsule and tubular lumen and ingested in relatively normal renal tubular epithelium (arrow in Fig. 2A). 8-OH-dG was strongly stained in renal tubular epithelium in which cell nuclei were strongly stained (arrow in Fig. 2B). ORP-150 was observed in intraglomerular cells and renal tubular epithelium (arrow in Fig. 2C).

2.4. Toxicological analysis

Screening for drugs was performed with whole blood and urine specimens. Samples were prepared by liquid–liquid extraction, and drugs were detected by GC–NPD and GC/MS.

Quantification for drugs was performed with whole blood. Promazine was used as the internal standard for the simultaneous quantification of promethazine and chlorpromazine. Hexobarbital was used as the internal standard for the quantification of phenobarbital. Samples were prepared by liquid–liquid extraction. The drugs were then detected by GC/MS-SIM of characteristic ions.

On the screening for basic drugs by liquid–liquid extraction, caffeine, phenobarbital, promethazine, chlorpromazine and chlorpromazine sulfoxide were detected in blood. Caffeine, phenothiazine, norpromethazine, promethazine, chlorophenothiazine, chlorpromazine, chlorpromazine sulfoxide and levomepromazine were detected in urine. By on-screening for acidic drugs using liquid–liquid extraction, phenobarbital was detected in blood. Caffeine, phenobarbital and hydroxyphenobarbital were detected in urine.

The results of the quantification of phenobarbital, promethazine and chlorpromazine levels in blood are shown in Table 1. The concentration of phenobarbital ranged from therapeutic to toxic levels, that of promethazine ranged from toxic to lethal levels and that of chlorpromazine was toxic.

2.5. Genetic analysis

For the genetic analysis, genomic DNA was isolated from blood samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The mutational analysis was performed using direct sequencing. All PCR products were sequenced directly on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

The *RYR1* gene contains 106 exons. Intronic primers for amplification from genomic DNA were designed for each exon within the three mutational hot-spot regions (exons 2–17, 39–46, 85–103). Mutations of the *CPT II* gene were analyzed according to the method of Kaneoka et al.[7] The *VLCAD* gene contains 20 exons, and we designed primer pairs for all its exons. CYP2C19, a metabolic enzyme for phenobarbital, was analyzed according to the method of Morita et al.[15] CYP2D6, a metabolic enzyme for promethazine and chlorpromazine, was analyzed using primer pairs specific to the *CYP2D6* gene.

Table 2
Identified mutations.

Gene	Nucleotide change	Exon(s)	Amino acid change	Zygosity
RYR1	^{7089}C > T	44	Silent mutation	Hetero
	^{7098}C > T	44	Silent mutation	Hetero
CPT II	^{1203}G > A	4	^{368}Val > Ile	Hetero
VLCAD	None	–	–	–
CYP2D6	^{1661}G > C	3	Silent mutation	Hetero
	^{2850}C > T	6	^{296}Arg > Ser	Hetero
	^{4180}G > C	9	^{486}Ser > Thr	Hetero
CYP2C19	None	–	–	–

The results of the genetic analysis are shown in Table 2. Genomic sequencing of the *RYR1* gene confirmed the silent mutations ^{7089}C > T (rs2228071) and ^{7098}C > T (rs2229147) in exon 44; however, there was no mutation causing an amino acid substitution in this subject. In the *CPT II* gene, the subject was found to be heterozygous for an amino acid substitution in exon 4, ^{1203}G > A causing a ^{368}Val > Ile substitution. Mutations of the *VLCAD* and *CYP2C19* genes were not found. In the *CYP2D6* gene, the subject was heterozygous for ^{1661}G > C, ^{2850}C > T and ^{4180}G > C, causing a silent mutation, ^{296}Arg > Ser and ^{486}Ser > Thr, respectively, in the *CYP2D6*∗*2* allele.

3. Discussion

The cadaver showed considerable salivation and nasopharyngeal discharge; so, death was suspected to be due to drug intoxication. Because the upper epidermis of some parts of the body had macerated, the subject was considered to be hyperhidrotic. Breakdown of skeletal muscle and protein leakage out of muscle cells were suspected because of discoloration of skeletal muscle, high levels of myoglobin in urine and pathological findings. The amount of urine retained in the bladder suggested that the urine flow was blocked. A biochemical analysis of the urine showed some renal disfunction. Histopathologically, myoglobin was observed in renal tubules; so, there is the probability that myoglobin was reabsorbed in the tubules. The expression of ORP-150 may have been induced by ischemic change accompanying acute renal failure in the agonal stage. Immunoreactions of 8-OH-dG and SOD to the peroxidative damage induced by myoglobin were suspected.[16,17] Based on these findings, it was considered that the myoglobinuria induced a deterioration in kidney function and then acute renal failure occurred. The toxicological analysis revealed components of vegetamin and metabolic substances. Concentrations of drugs in blood did not reach a lethal level. Histologically, lungs showed severe congestion and edema, these findings were considered as one of the signs of intoxication. Heart was slightly hypertrophic; however, it is not enough to be the cause of death. Based on the overall findings, the cause of death was considered to be vegetamin-induced rhabdomyolysis.

We performed a mutational analysis of 5 genes in this subject. In the *RYR1* gene, there were the silent mutations ^{7089}C > T (rs2228071) and ^{7098}C > T (rs2229147) in exon 44, but there was no mutation causing an amino acid substitution in the three hot-spot regions. Most polymorphisms associated with the risk of disease are not nonsynonymous. For example, a silent polymorphism

Table 1
The results of the toxicological analysis of blood.

	Blood level (μg/mL)	Therapeutic level	Toxic level	Lethal level
Phenobarbital	38.2	10–40	40–60	>80
Promethazine	2.22	0.1–0.4	1–2	2.4–12
Chlorpromazine	0.96	0.05–0.5	0.5–2	3–35

in the *MDR1* gene affects substrate specificity.[18] Although there has been no report of an association rs2228071 or rs2229147 with malignant hyperthermia, there is a possibility that rs2228071 or rs2229147 affects in vivo protein function.

In the *CPT II* gene, there was one heterozygous amino acid substitution in this subject. However, it has been reported that the V368I substitution alone did not affect enzyme activity in vitro.[19] Although the effect of V368I on CPT II activity in vivo remains unclear, there is a possibility that the CPT II activity in this subject was normal or near normal levels.

The *CYP2D6* gene is highly polymorphic, causing no, decreased, normal or increased enzyme activity.[20] A relationship between increased drug concentrations and rhabdomyolysis has been reported.[21] The CYP2D6*4 polymorphism is associated with statin-induced muscle effects.[22] The subject was heterozygous for the *CYP2D6*1* allele and *CYP2D6*2* allele. CYP2D6*2 exhibits metabolic activity similar to that of the wild type for various substrates.[23] Therefore, the metabolism of promethazine and chlorpromazine was considered to be nearly normal in this subject. Genetic polymorphisms of CYP2C19 play an important role in the pharmacokinetic variability of phenobarbital.[24] However, there was no mutation in the *CYP2C19* gene in this subject. Therefore, it was inferred that vegetamin was metabolized nearly normally in this subject. Adult-onset VLCAD deficiency is a rare but important cause of rhabdomyolysis.[10] But there was no mutation, and his VLCAD function was considered to be normal.

Many drugs may alter myocyte function, resulting in rhabdomyolysis. Rhabdomyolysis is a potentially lethal syndrome resulting from the lysis of muscle fibers with leakage of potentially toxic cellular contents into the systemic circulation. Rhabdomyolysis is extremely rare in people taking the most commonly prescribed drugs. Individual differences in muscle cell fragility and metabolic enzymes of offending drugs may be involved in the development of rhabdomyolysis.

In the present case, a man and his female cohabiter both took almost equal numbers of vegetamin tablets but only he died. It was suspected that he was more sensitive to rhabdomyolysis than his cohabitor. In the five genes examined in this study, he showed three mutations including two silent mutations in two genes. In forensic autopsy cases, if the concentration of a drug in blood is lower than lethal level, it is difficult to determine the cause of death. As the influence of drugs differ widely among individuals, it is necessary to consider the genetic background. In forensic autopsy cases, because there is no antemortem information, suspected toxicological death should be analyzed by conducting not only toxicological but also genetic tests.

Conflict of Interest

There are no conflicts of interest.

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Ethical Approval

The study was approved by the Fukuoka University School of Medicine Ethical Review Board (reference number: 328).

References

1. Richards JR. Rhabdomyolysis and drugs of abuse. *J Emerg Med* 2000;**19**:51–6.
2. Phillips MS, Fujii J, Khanna VK, et al. The structural organization of the human skeletal muscle ryanodine receptor (RYR1) gene. *Genomics* 1996;**34**: 24–41.
3. Robinson R, Carpenter D, Shaw MA, Halsall J, Hopkins P. Mutations in RYR1 in malignant hyperthermia and central core disease. *Hum Mutat* 2006;**27**: 977–89.
4. Davis M, Brown R, Dickson A, et al. Malignant hyperthermia associated with exercise-induced rhabdomyolysis or congenital abnormalities and a novel RYR1 mutation in New Zealand and Australian pedigrees. *Br J Anaesth* 2002;**88**:508–15.
5. Wappler F, Fiege M, Steinfath M, et al. Evidence for susceptibility to malignant hyperthermia in patients with exercise-induced rhabdomyolysis. *Anesthesiology* 2001;**94**:95–100.
6. Bieber LL. Carnitine. *Annu Rev Biochem* 1988;**57**:261–83.
7. Kaneoka H, Uesugi N, Moriguchi A, et al. Carnitine palmitoyltransferase II deficiency due to a novel gene variant in a patient with rhabdomyolysis and ARF. *Am J Kidney Dis* 2005;**45**:596–602.
8. Aoki J, Yasuno T, Sugie H, et al. A Japanese adult form of CPT II deficiency associated with a homozygous F383Y mutation. *Neurology* 2007;**69**:804–6.
9. Vianey-Saban C, Divry P, Brivet M, et al. Mitochondrial very-long-chain acyl-coenzyme A dehydrogenase deficiency: clinical characteristics and diagnostic considerations in 30 patients. *Clin Chim Acta* 1998;**269**:43–62.
10. Voermans NC, van Engelen BG, Kluijtmans LA, Stikkelbroeck NM, Hermus AR. Rhabdomyolysis caused by an inherited metabolic disease: very long-chain acyl-CoA dehydrogenase deficiency. *Am J Med* 2006;**119**:176–9.
11. Hargraves JAHW, Racha JK, Levy RH. Identification of enzymes responsible for the metabolism of phenobarbital [abstract]. *Int Soc Stud Xenobiotics Proc* 1996;**10**:259.
12. Nakamura K, Yokoi T, Inoue K, et al. CYP2D6 is the principal cytochrome P450 responsible for metabolism of the histamine H1 antagonist promethazine in human liver microsomes. *Pharmacogenetics* 1996;**6**:449–57.
13. Muralidharan G, Cooper JK, Hawes EM, Korchinski ED, Midha KK. Quinidine inhibits the 7-hydroxylation of chlorpromazine in extensive metabolisers of debrisoquine. *Eur J Clin Pharmacol* 1996;**50**:121–8.
14. Tsukamoto Y, Kuwabara K, Hirota S, et al. Expression of the 150-kd oxygen-regulated protein in human breast cancer. *Lab Invest* 1998;**78**:699–706.
15. Morita J, Kobayashi K, Wanibuchi A, et al. A novel single nucleotide polymorphism (SNP) of the CYP2C19 gene in a Japanese subject with lowered capacity of mephobarbital 4′-hydroxylation. *Drug Metab Pharmacokinet* 2004;**19**:236–8.
16. Ishigami A, Tokunaga I, Gotohda T, Kubo S. Immunohistochemical study of myoglobin and oxidative injury-related markers in the kidney of methamphetamine abusers. *Leg Med (Tokyo)* 2003;**5**:42–8.
17. Tokunaga I, Kubo S, Ishigami A, Gotohda T, Kitamura O. Changes in renal function and oxidative damage in methamphetamine-treated rat. *Leg Med (Tokyo)* 2006;**8**:16–21.
18. Kimchi-Sarfaty C, Oh JM, Kim IW, et al. A "silent" polymorphism in the MDR1 gene changes substrate specificity. *Science* 2007;**315**:525–8.
19. Taroni F, Verderio E, Fiorucci S, et al. Molecular characterization of inherited carnitine palmitoyltransferase II deficiency. *Proc Natl Acad Sci USA* 1992;**89**: 8429–33.
20. Dorado P, Penas-Lledo EM, Llerena A. CYP2D6 polymorphism: implications for antipsychotic drug response, schizophrenia and personality traits. *Pharmacogenomics* 2007;**8**:1597–608.
21. Omar MA, Wilson JP, Cox TS. Rhabdomyolysis and HMG-CoA reductase inhibitors. *Ann Pharmacother* 2001;**35**:1096–107.
22. Frudakis TN, Thomas MJ, Ginjupalli SN, Handelin B, Gabriel R, Gomez HJ. CYP2D6*4 polymorphism is associated with statin-induced muscle effects. *Pharmacogenet Genomics* 2007;**17**:695–707.
23. Sakuyama K, Sasaki T, Ujiie S, et al. Functional characterization of 17 CYP2D6 allelic variants (CYP2D6.2, 10, 14A-B, 18, 27, 36, 39, 47-51, 53-55, and 57). *Drug Metab Dispos* 2008;**36**:2460–7.
24. Yukawa E, Mamiya K. Effect of CYP2C19 genetic polymorphism on pharmacokinetics of phenytoin and phenobarbital in Japanese epileptic patients using non-linear mixed effects model approach. *J Clin Pharm Ther* 2006;**31**:275–82.